

Antibiogram and Heavy Metal Resistance Pattern of *Salmonella* spp. Isolated from Wild Asian Sea Bass (*Lates calcarifer*) from Tok Bali, Kelantan, Malaysia

Lee S. Wei ^{1,*} and Wendy Wee ²

¹Department of Agro Industry, Faculty of Agro Industry and Natural Resources, Universiti Malaysia Kelantan, Pengkalan Chepa, 16100, Kota Bharu, Kelantan, ²Department of Fisheries Science and Aquaculture, Faculty of Agrotechnology and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia

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Abstract

The aim of this study is to characterize antibiogram and heavy metal resistance pattern of *Salmonella* spp. isolated from wild Asian sea bass (*Lates calcarifer*). *Salmonella* spp. is recognized as a food borne pathogen to humans. Therefore, this study was carried out to determine the suitable antibiotic in controlling *Salmonella* spp. isolated from Asian sea bass, Malaysian favorite seafood. In the present study, *Salmonella* spp. was isolated using Xylose Lysine Desoxycholate (XLD) medium. The bacterial isolates were then identified using conventional biochemical tests and conformed to commercial identification kit. A total of 150 isolates of *Salmonella* spp. were randomly selected for antibiotic and heavy metal tolerance tests. Fourteen antibiotics, namely oxolinic acid (2 µg), ampicillin (10 µg), erythromycin (15 µg), furazolidone (15 µg), lincomycin (15 µg), colistin sulfate (25 µg), oleandomycin (15 µg), doxycycline (30 µg), nitrofurantoin (50 µg), fosfomicin (50 µg), florfenicol (30 µg), flumequine (30 µg), tetracycline (30 µg), and spiramycin (100 µg) and four heavy metals; mercury (Hg²⁺), cadmium (Cd²⁺), chromium (Cr⁶⁺) and copper (Cu²⁺) were tested in the present study. The results of the present study indicating that oxolinic acid were found the most effective in controlling present bacterial isolates in which 85.3 % of the present bacterial were sensitive to them. This was followed by tetracycline and nitrofurantoin in which 85.2 % of the bacterial isolates were sensitive to it. On the other hand, all the bacterial isolates were resistant to lincomycin and oleandomycin. The findings of the present study indicate that the samples may be highly exposed to the tested antibiotics and heavy metals.

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1. Introduction

Food poisoning is a case that occurs when a patient is exposed to or consumes food or beverage contaminated with bacteria, parasites, viruses, or a toxin produced by microorganism. *Salmonella* spp. is one of the pathogens that are recognized as a causative agent of food poisoning. Recently, a local newspaper in Malaysia has reported that food poisoning cases of Kota Bharu, Kelantan, Malaysia increased 100% and the sampling sites of the present study were only about 100 km away from Kota Bharu. Salmonellosis due to *Salmonella* spp. was recognized as a public health problem associated with a significant morbidity and mortality in those infected with the pathogens (Lunestad *et al.*, 2007). These bacteria are ubiquitous and are reported to be commonly found in food products and water samples (Baudart *et al.*, 2000). Products such as fish meal, meat, bone meal, maize and soy products may be contaminated with this bacterium at high prevalence (Jones and Richardson, 2004). Thus, fish

may be one of the sources of *Salmonella* spp. harboring in the cultured fish from aquaculture sites. Although *Salmonella* spp. was not recognized as fish pathogen, this bacterium was reported as persistently found in the gastro intestinal, internal organs and muscle of fish after the fish was exposed orally to high dose to this bacterium (Hagen, 1966; Buras *et al.*, 1985). Therefore, this paper discusses the presence of *Salmonella* spp. in the wild of *Lates calcarifer* as well as their antibiogram and heavy metal resistance pattern. Furthermore, the implications of such *Salmonella* contamination on fish and human health are assessed.

2. Materials and Methods

A total of 50 of wild caught Asian sea bass, *Lates calcarifer* with the size 20 to 25 cm were sampled at Tok Bali seaside, Kelantan, Malaysia. The water parameters of the sampling sites were measured using pH meter (YSI, USA). The temperature, dissolved oxygen, pH, and salinity of the sampling sites were 29.52 °C, 6.87 mg/l, 8.81, and 28.31 ppt, respectively.

A total of 10 g of the minced flesh of *L. calcarifer* was diluted in 100 ml sterile physiological saline followed by a

* Corresponding author: leeseongwei@yahoo.com

serial dilution in sterile physiological saline and plated on two types of medium; Tryptic Soy Agar (TSA) and Xylose Lysine Desoxycholate (XLD) (Merck, Germany). All the inoculated media were incubated at room temperature for 24 to 48 h. The bacterial colonies that grew on the selective media were further selected for the identification test. The bacterial isolates were identified using conventional biochemical tests (Holt *et al.*, 1994) and conformed to commercial identification kit (BBL, USA). All the bacterial isolates were identified as *Salmonella* spp.

The isolates (n = 150) were cultured in Tryptic Soy Broth (TSB) (Oxoid, England) for 24 h at room temperature. The bacterial cells were then centrifuged at 14,500 rpm for 5 min by using minispin (Eppendorf, Germany). The concentration of the bacterial cells were adjusted to 10^6 colony forming unit (CFU) using saline and monitored with Biophotometer (Eppendorf, Germany) before being swabbed onto the prepared Mueller Hinton Agar (MHA) (Oxoid, England). Antibiotic susceptibility test was conducted according to Kirby–Bauer disk diffusion method using MHA (Bauer *et al.*, 1966). Antibiotics tested were: OA2; oxolinic acid (2 µg/disk), F50; nitrofurantoin (50 µg/disk), AMP10; ampicillin (10 µg µg/disk), E15; erythromycin (15 µg/disk), FR15; furazolidone (15 µg/disk), MY10; lincomycin (10 µg/disk), CT25; colistin sulfate (25 µg/disk), OL30; oleandomycin (30 µg/disk), FOS50; fosfomycin (50 µg/disk), DO30; doxycycline (30 µg/disk), FFC30; florfenicol (30 µg/disk), UB30; flumequine (30 µg/disk), TE30; tetracycline (30 µg/disk) and SP100; spiramycin (100 µg/disk) (Oxoid, England). Interpretation of the results, namely sensitive (S), intermediary sensitive (I) and resistance (R), was made in accordance to the standard measurement of inhibitory zones in millimeter (mm) (CLSI, 2006).

Multiple antibiotic resistance (MAR) index of the isolates against the tested antibiotics was calculated based on the following formula (Sarter *et al.*, 2007; Lee *et al.*, 2009; Lee *et al.*, 2010):

MAR index (multiple antibiotic resistance) = $X / (Y \times Z)$

X = total number of antibiotic resistance cases

Y = total number of antibiotics used in the study

Z = total number of bacterial isolates

A MAR index value of equal or less than 0.2 was defined as those antibiotics that were rarely or never used for the animal in terms of treatment, but if the MAR index value was higher than 0.2 it is considered as that when the animals have received high risk of exposure to those antibiotics.

Heavy metal resistance test was carried out as described by Miranda and Castillo (1998). Bacterial tolerance to four elements of heavy metal: mercury (Hg^{2+}), cadmium (Cd^{2+}), chromium (Cr^{6+}) and copper (Cu^{2+}) was determined by agar dilution method. Overnight bacterial suspension was spread onto plates of TSA medium incorporated with different concentrations of $HgCl_2$, $CdCl_2$, $K_2Cr_2O_7$, and $CuSO_4$ (Fluka, Spain). By two-fold dilutions, concentration of both Cd^{2+} and Cr^{6+} ranged from 25 to 400 µg/mL while concentration of Hg^{2+} and Cu^{2+} ranged from 2.5 to 40 µg/mL and 150 to 2400 µg/mL, respectively. For the purpose of defining metal resistance, the isolates were

considered resistant if growth was obtained at concentrations of 10 µg/mL (Hg^{2+}), 100 µg/mL (Cd^{2+} and Cr^{6+}), and 600 µg/mL (Cu^{2+}), respectively (Allen *et al.*, 1977). The operational definition of tolerance used in this study was based on the positive bacterial growth when the concentration of each heavy metal was above the stated concentration for resistance.

3. Results

The total plate count of *Salmonella* spp. from the water sample in the Asian sea bass hatchery was 1.0×10^3 colony forming unit (CFU)/ml. In the present study, all bacterial isolates were found resistant to lincomycin and oleandomycin. However, more than 46 % of the bacterial isolates were found to be resistant to fosfomycin, furazolidone, ampicillin, doxycycline and spiramycin (Fig. 1). On the other hand, more than 70% of the bacterial isolates were sensitive to nitrofurantoin, tetracycline, and oxolinic acid. More than 50% of the present bacterial isolates were sensitive to colistin sulfate, erythromycin, and florfenicol. Overall, in the antibiotic susceptibility test, 49.5 % was recorded as antibiotic resistance case and 4.8% and 45.7 % was recorded as intermediary sensitive and sensitive case. The MAR value of the present study was 0.50. With regards to the heavy metal tolerance test, 66.7% and 73.3% of present bacterial isolates were sensitive to Cd^{2+} and Cu^{2+} respectively. 33.3 % of the bacterial isolates were sensitive to Hg^{2+} . All the bacterial isolates were found to be resistant to Cr^{6+} .

4. Discussion

The results of the present study reveal the existence of *Salmonella* spp. in the flesh of the Asian sea bass. However, the population of the bacteria in the sample is still within the safety level for human consumption. As long as the hygiene procedure was taken during the food preparation, the risk of transmission of *Salmonella* spp. to humans via fish products from that sampled area is minimal. In the present study, 46.7% of *Salmonella* spp. was found to be resistant to florfenicol. This may due to the bacterial strains that developed a florfenicol resistance gene in their genomic property. The existence of this florfenicol resistance gene was detected as early as 1969; this gene was found in a plasmid of *Klebsiella pneumoniae* in France (Smith, 2008). Furthermore, it was found in *S. enterica* serovar *typhimurium* DT 104 in the United States in 1985 (Smith, 2008), and in a plasmid of *Vibrio damsela* that infected fish farms in Japan in 1990s (Kim *et al.*, 1993). Hence, we may conclude that this antibiotic resistance gene can be found in various species of bacteria. Subsequently, the incidence of florfenicol resistance was widely spread among bacteria species including the bacterial strains in the present study. All the present bacterial isolates were found resistant to oleandomycin and lincomycin. Therefore, we suggested that these two types of antibiotics may be used as supplement for *Salmonella* spp. isolation medium to inhibit the growth of other microorganisms such as fungal.

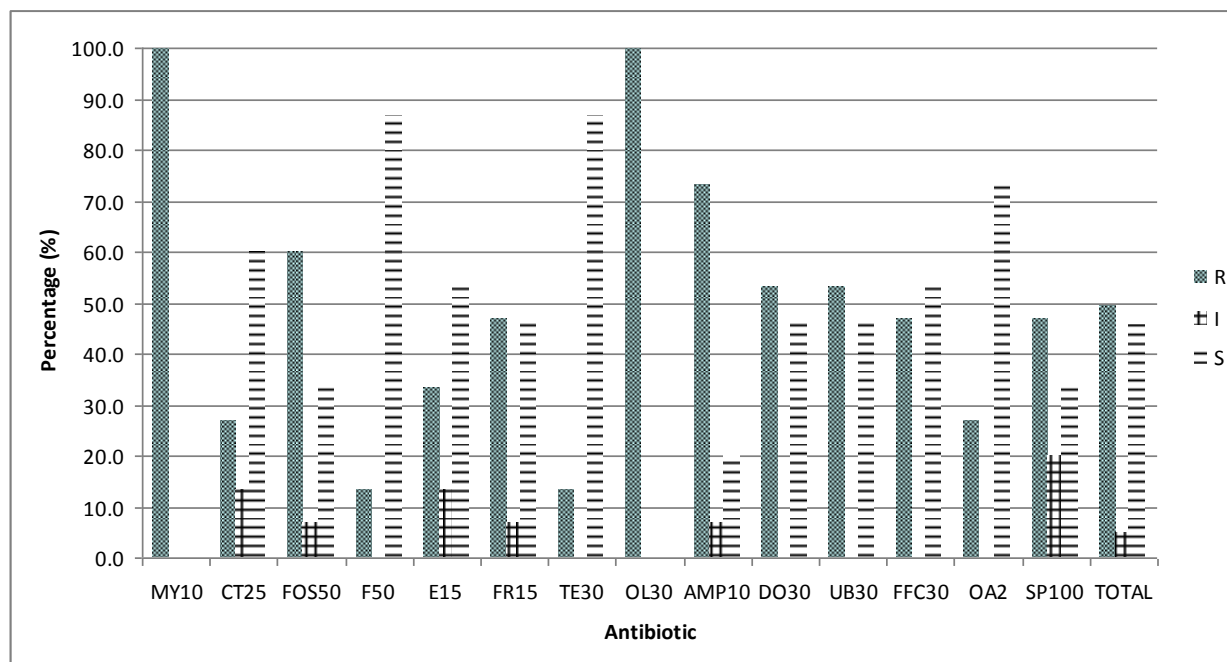


Figure 1. Antibiotic sensitivity of *Salmonella* spp. isolated from wild Asian sea bass.

OA2; oxolinic acid (2 µg/disk), F50; nitrofurantoin (50 µg/disk), AMP10; ampicillin (10 µg µg/disk), E15; erythromycin (15 µg/disk), FR15; furazolidone (15 µg/disk), MY10; lincomycin (10 µg/disk), CT25; colistin sulfate (25 µg/disk), OL30; oleandomycin (30 µg/disk), FOS50; fosfomycin (50 µg/disk), DO30; doxycycline (30 µg/disk), FFC30; florfenicol (30 µg/disk), UB30; flumequine (30 µg/disk), TE30; tetracycline (30 µg/disk) and SP100; spiramycin (100 µg/disk)

Furthermore, oleandomycin was recognized as fungicidal antibiotic as early as 1958 (Holzer, 1958). Several studies reported that most of the bacterial isolated obtained from aquatic animals were found highly resistant to oleandomycin and lincomycin. For instance, Lee *et al* (2009a) claimed that *Vibrio* spp. isolated from *Litopenaues vannamei* were highly resistant to these two types of antibiotics. Similar finding was also observed in the study of Lee *et al.* (2009b) (bacteria isolated from giant freshwater prawn), Lee *et al.* (2009c) (bacteria isolated from Asian seabass) and Lee *et al.* (2009d) (bacteria isolated from American bullfrog). A similar resistance pattern was also documented of bacteria isolates from freshwater Asia seabass fingerling Lee *et al.* (2010a) and silver catfish and red hybrid tilapia (Lee *et al.*, 2010b). Hence, we may conclude that most of the bacterial isolates from aquatic animals were found resistant to oleandomycin and lincomycin. The high value of MAR that was observed in the present study indicates that the fish of the present study was under high risk of being exposed to the tested antibiotics in which the result can give us information on the existence or contamination of the tested antibiotics residues in the sampling areas.

In addition to antibiotic test, however, a low percentage of the present bacterial isolates was resistant to Cd²⁺ and Cu²⁺, but a high percentage of the present bacterial isolates was resistant to Hg²⁺ and Cr⁶⁺. This indicates that the sampling areas in our study may have been exposed to these heavy metal residues and this may be due to agricultural activities that surrounded the sampled area in which the discharged agricultural wastes such as fertilizer may contain heavy metal residues. Many studies on the

heavy metal resistance pattern of bacteria isolated from various aquatic animals namely Giant freshwater prawn, *Macrobrachium rosenbergii* (Lee *et al.*, 2009a), Asian seabass, *Lates calcarifer* (Lee *et al.*, 2009b), American bullfrog, *Rana catesbeiana* (Lee *et al.*, 2009c), Silver catfish, *Pangasius sutchi* (Lee *et al.*, 2010a) and red hybrid tilapia, *Tilapia* sp (Lee *et al.*, 2010b) were reported in the literature.

In conclusion, the results of the present study indicate that the sampling areas may be contaminated with the tested antibiotic and heavy metal residues. Therefore, we proposed that further study should be carried out in the near future to reveal the actual status of contamination of the sampling sites before we can come to a final decisive conclusion.

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